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Review

Glutamate transporters in brain ischemia: to modulate or not?

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In this review, we briefly describe glutamate (Glu) metabolism and its specific transports and receptors in the central nervous system (CNS). Thereafter, we focus on excitatory amino acid transporters, cystine/glutamate antiporters (system x_c^-) and vesicular glutamate transporters, specifically addressing their location and roles in CNS and the molecular mechanisms underlying the regulation of Glu transporters. We provide evidence from *in vitro* or *in vivo* studies concerning alterations in Glu transporter expression in response to hypoxia or ischemia, including limited human data that supports the role of Glu transporters in stroke patients. Moreover, the potential to induce brain tolerance to ischemia through modulation of the expression and/or activities of Glu transporters is also discussed. Finally we present strategies involving the application of ischemic preconditioning and pharmacological agents, *eg* β-lactam antibiotics, amitriptyline, riluzole and N-acetylcysteine, which result in the significant protection of nervous tissues against ischemia.

Keywords: glutamate; brain ischemia; excitatory amino acid transporter; cystine/glutamate antiporter; vesicular glutamate transporter; ischemic preconditioning; ceftriaxone

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Introduction

Glutamate (Glu) is the most widespread neurotransmitter in the central nervous system (CNS) and is involved in almost every aspect of physiological brain functioning, including memory, learning, cognition, and the control of emotion[1]. The estimated concentration of Glu in the entire brain is approximately 5–15 mmol per kg wet weight (depending on the brain region), but only a small part of the Glu is released into the synaptic cleft[2]. Glu is almost exclusively (99.99%) present intracellulary, where the intracellular Glu concentration ($[Glu]_i$) is within 1–10 mmol/L, with the largest concentration observed in synaptic vesicles at the nerve terminals (ca 100 mmol/L), whereas the extracellular Glu concentration ($[Glu]_e$) is only a few μmol/L[1]. Glu is exocytotically released from both astrocytes and neurons, which possess membrane receptors, susceptible to Glu excitation[3]. Even though Glu is a predominant neurotransmitter, this compound can be toxic. Although under physiological conditions the majority of Glu is stored intracellulary, under some pathological conditions, the extracellular concentration is dramatically increased. Neuronal injury caused by brain ischemia is mediated through a massive overload with Glu, Glu receptor activation, and Ca^{2+}-dependent excitoxicity[4]. The $[Glu]_e$ is highest just after the onset of ischemia, while the mechanisms underlying the uncontrolled release of Glu are complex. Apart from the dysfunction of excitatory amino acids transporters (EAATs), these mechanisms involve the excessive release of Glu and post-necrotic Glu liberation. Depending on the severity of brain injury, the overactivation of Glu receptors might cause necrosis or apoptosis[5]. Because of the risk of excitotoxic damage, the precise physiological control must maintain the homeostasis. EAATs localized to both astrocytes and neurons play a major role in system regulation. In this review, we discuss the potential modulation of Glu transporters in the context of brain ischemia and tolerance to this condition. The nomenclature of particular Glu transporters is different for animals and humans. In humans, EAAT1, EAAT2, and EAAT3 are the terms used for these transporters, whereas in rodents GLAST, GLT-1, and EAAC1 are used, respectively. In this review, we use the latter terminology because the majority of the studies cited here were conducted on rodents.

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Glutamate metabolism and cycling

There are only a few ways for the body to produce Glu mol-

ecules: 1) the Glu-glutamine cycle; 2) synthesis in neurons and astrocytes from glucose; and 3) synthesis inside neurons from lactate delivered from astrocytes. A fraction of Glu present in the brain participates in the Glu-glutamine cycle in neurons and astrocytes. However, *de novo* synthesis is necessary because Glu can be oxidized and cannot be entirely regenerated through this cycle. Glu also does not cross the blood brain barrier and hence is not delivered to the CNS through the ingestion of food. Glucose is the major substrate for Glu synthesis in astrocytes and neurons[6], and the influx of Na^+ (together with Glu through EAATs) stimulates glucose uptake in astrocytes and neurons[7]. Glucose enters the tricarboxylic acids cycle (TCA cycle) and provides α-ketoglutarate (α-KG) as a carbon backbone of Glu, while the source of the nitrogen used in Glu synthesis is leucine, alanine, isoleucine or asparate[8]. Glu in neurons can also be synthesized from lactate delivered from astrocytes. In some neurons, Glu might be converted to gamma-aminobutyric acid (GABA) through the action of Glu decarboxylase[9]. Glu is released from presynaptic neurons into the synaptic cleft, where this compound binds to specific ionotropic (iGluR) or metabotropic receptors (mGluR) located on postsynaptic and presynaptic neurons and glial cells. $[Glu]_e$ can also be elevated through the cystine-Glu antiporter (system x_c-)[10]. Because there is no evidence of the extracellular metabolism of Glu and because high Glu concentrations are highly toxic, the mechanism responsible for Glu clearance must be efficient[11]. Through GLAST and GLT-1, primarily located on astrocytes[12, 13], Glu is taken up through the inward co-transport of three Na^+ molecules, one H^+ molecule and the counter transport of one K^+ [14, 15]. The Na^+-dependent neuronal Glu transporters include EAAC1 and EAAT4, which exhibit a similar mechanism of action. In astrocytes, Glu is converted to glutamine through glutamine synthetase (a specific enzyme in astrocytes and oligodendrocytes) in an ATP-dependent process. Notably, not every molecule of Glu is converted to glutamine, as a small fraction of Glu is degraded to α-KG and enters the TCA cycle. The glutamine produced from astrocytes is released through the glutamine transporter system N transporter 1 (SN1) and reaches neurons *via* system A transporter (SAT1). Here, glutamine is converted to Glu through phosphate-activated glutaminase[16]. Subsequently, transmitter is loaded into vesicles through vesicular glutamate transporters (VGLUTs), and after interaction with soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), Glu is released into the synaptic cleft where it binds to Glu receptors and EAATs. Thereafter, the Glu-glutamine cycle terminates (Figure 1)[17].

Glutamate neurotransmission and intracellular signaling

Glu is released from presynaptic terminals through two different Ca^{2+}-dependent or Ca^{2+}-independent mechanisms. The first mechanism involves N-type and P/Q-type voltage-dependent Ca^{2+} channels (VDCC)[18]. Ca^{2+}-independent release is mediated through the reverse activity of Glu transporters[19]. Glu acts on two types of receptors, iGluR and mGluR. The activity of iGluR depends on ion influx. iGluRs are ion channels permeable to Ca^{2+} and Na^+ and primarily localized to postsynaptic membranes. However, some subtypes of *N*-methyl-*D*-aspartate receptor (NMDA) are extrasynaptically present. NMDA receptors have a tetrameric structure, comprising two GluN1 subunits and at least one GluN2 (A, B, C, or D) or GluN3 (A or B). The formation of the distinct receptors depends on many factors, such as the development regulation and localization of specific neurons. NMDA receptors are characterized according to their affinity for Glu, regulated through binding sites for Mg^{2+}, Zn^{2+}, H^+, glycine, and polyamines[20]. The excessive stimulation of extrasynaptic NMDA receptors might lead to apoptosis signaling and consequent cell death. Nevertheless, the stimulation of synaptic NMDA receptors might promote cell survival through the Ca^{2+}-dependent signal transduction pathway[21-23]. However, recent studies have shown that neurotoxic effects are primarily mediated through synaptic NMDA receptors and that the silencing of extrasynaptic NMDA receptors does not exert any protective effect. In these studies, synaptic, but not extrasynaptic, NMDA receptors were involved in long-term potentiation, and in long-term depression both types of receptors are activated[24]. The hyperactivation of NMDA receptors is the most important mechanism of excitotoxic Glu-induced neuron damage[25]. However, other pathways, dependent on the activity of 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid/kainate receptors (AMPA/KA receptors), or mGluR are also important. The stimulation of Glu receptors increases the intracellular Ca^{2+} concentration through NMDA receptors and VDCC, Ca^{2+} release from internal stores, and the reversal of the Na^+/Ca^{2+} exchanger (NCX)[26]. Overloading the intracellular space with Ca^{2+} results in the dysfunction of mitochondria, activation of caspases and calpains, increased production of nitrogen oxide (NO) and reactive oxygen species, and the activation of the arachidonic acid pathway. The progression of these processes results in neuronal cell death through apoptosis or necrosis[27, 28]. AMPA receptors are characterized by the fast generation of excitatory postsynaptic potential and lower affinity to Glu. The short-term binding of Glu to AMPA receptors induces rapid transmission but also rapid current decay. The long-term binding of NMDA receptors to Glu, as a consequence of high affinity to Glu, produces prolonged signaling and delayed current decay. AMPA and kainate (KA) receptors also have tetrameric structures and comprise GluA1–4 and GluK1–5 subunits, respectively. mGluR acts via G-proteins. At least eight subtypes of mGluR, mGluR1–8, have been identified, and each receptor subtype is characterized by different molecular and pharmacological properties. The metabotropic Glu receptor family is divided into three groups: group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7, and mGluR8). This classification is based on the similarity of the sequence, mode of the signal transduction, localization and pharmacology of particular receptor groups[29, 30]. Group I mGluRs are coupled to phospholipase C through $G_{q/11}$ protein, leading to the production of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol. This group is located postsynaptically near ionotropic recep-

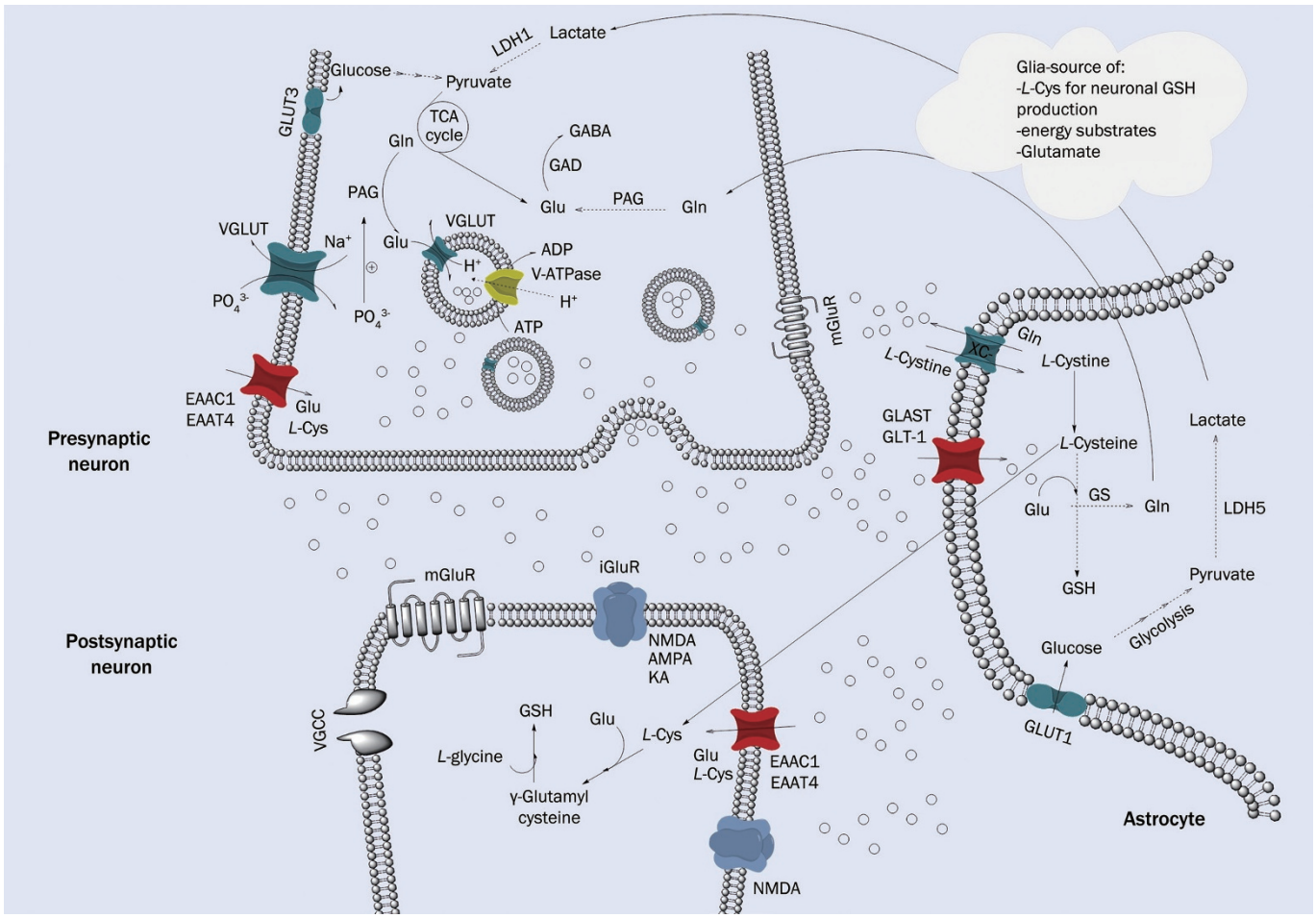

Figure 1. Glutamate cycling and metabolism. When Glu is released into synaptic cleft it binds to various synaptic and extrasynaptic receptors – mGluR and iGluR. While synaptic Glu concentration reaches excessive concentration EAATs start uptake direct into astrocytes (GLAST and GLT-1) and neurons (EAAC1 and EAAT4). Inside astrocytes Glu is subjected into Glu–Gln cycle. First, in glial cell it is converted to glutamine by GS and is directed by means of specific transporters towards neurons. In neurons Gln is reversed to Glu by phosphate activated glutaminase (phosphate is delivered by VGLUTs, the second type of activity of plasma membrane VGLUTs) and by co-operation of VGLUT and V-ATPase is packed into synaptic vesicles, which after interaction with SNARE proteins (not shown on the figure) are released into synaptic cleft and Glu-Gln cycle is closed. However, this mechanism is not efficient enough to maintain constant amount of Glu. Another source of Glu is glucose entering astrocytes and neurons by GLUT1 & GLUT3, respectively. Glucose as a substrate of glycolysis forms pyruvate which after enzymatic reaction catalized by LDH is converted to lactate. From astrocytes lactate diffuse to neurons where after few enzymatic reaction of TCA cycle forms Glu. On this figure there is also demonstrated mechanism of action of xc- system which is in fact cysteine/Glu antiporter. By means of this transporter *L*-cystine is uptaken from synaptic space to astrocyte where is converted to *L*-Cys which may be transported to neurons and serve as a substrate to GSH synthesis, or this synthesis may take place in astrocyte. ADP indicates adenosine diphosphate; ATP, adenosine triphosphate; GLAST, GLT-1, EAAC1, and EAAT4, excitatory amino acid transporter 1–4 respectively; Gln, glutamine; Glu, glutamate; iGluR, ionotropic glutamate receptors; GLUT1 & GLUT3, glucose transporters; GS, glutamine synthetase; GSH, glutathione; *L*-Cys, *L*-cysteine; LDH1&5, lactate dehydrogenase 1&5; mGluR, metabotropic glutamate receptors; PAG, phosphate activated glutaminase; TCA cycle, tricarboxyl acids cycle; VGLUT, vesicular glutamate transporter; V-ATPase, vacuolar-type H^+-ATPase; System xc-/xCT, cystine/glutamate antiporter.

tors, such as NMDA and AMPA receptors, and probably presynaptically (according to data from studies on synaptoneurosomes)[31–36]. This localization induces the potentiation of Glu ionotropic receptor activity through mGluR1/5[37]. Groups II and III are negatively coupled to adenylate cyclase and are localized both pre- and postsynaptically, which determines the function of these receptors. Presynaptic receptors inhibit the release of Glu, while postsynaptically localized receptors interfere with the modulation of ion channels and release of other neurotransmitters[29, 37, 38]. mGluR5 and mGluR3 are present on the glial cells, particularly astrocytes[39, 40].

Glu transporters

Location in the brain

GLAST, GLT-1, and EAAC1 are widely distributed in the CNS, while EAAT4 and EAAT5 are predominantly expressed in the cerebellum and retina, respectively[41]. System x_c^- is specific to the border of the CNS[42], whereas VGLUTs are located

in several brain structures[43] (Table 1).

Function

The general function of EAATs is to regulate the extracellular Glu concentration and maintain the concentration of Glu at low physiological levels to avoid toxic effects. After release into the synaptic cleft, Glu is rapidly removed through EAATs into glial cells and neurons. The major transporter is the GLT-1, which is responsible for more than 90% of total Glu uptake[57]. Studies using antisense oligonucleotides directed against GLT-1 have revealed that the loss of GLT-1 results in a substantial increase in $[Glu]_e$[80]. GLASTs, expressed on Müller glial cells, regulate extracellular Glu levels in the retina[55, 81]. EAAC1 transports cysteine (along with Glu)[82], providing a substrate for glutathione (GSH) synthesis in neurons[84]. The role of the EAAT4 is to regulate neuronal excitability through counteracting the depolarization of neurons[41]. Both EAAT4 and EAAT5 possess a thermodynamically uncoupled Cl^- flux,

Table 1. Expression of the Glu transporters at the mRNA and protein levels in the CNS and in cellular cultures.

Glu transporter	Brain structures		Cells in the nervous system		Cell cultures	
	mRNA	Protein	mRNA	Protein	mRNA	Protein
GLAST	Cerebellum, cerebral cortex, hippocampus, striatum, thalamus, hypothalamus[44]	Throughout the CNS[45], retina[46]	Astrocytes, Müller cells, pigment epithelia[47, 48], rat cohlear hair cells (neurons)[49]	Bergman glia, Purkinje cells[45], Müller cells[46]	Astrocytes, microglia, oligodendrocytes[50], astrocytes, prepared from mouse cerebral cortex[51], neuronal PC12 line[52]	Rat hippocampal neurons, microglia[53]
GLT-1	Throughout the brain, except the cerebellum[54]	Throughout the CNS[55]	No data	Glial cells; astrocytes[56], neurons, oligodendrocytes-during development[57]	Astrocytes, microglia and oligodendrocytes[50]	Rat primary hippocampal and cortical neurons[58], C57BL/6J mice hippocampal microglia[59]
EAAC1	No data	Throughout the CNS[60]	No data	Neurons[55], oligodendrocytes[61]	No data	No data
EAAT4	Cerebellum, slight in cortex and retina[54]	Throughout the fore- and midbrain[62, 63], cortex, cerebellum- during development[64]	No data	Purkinje cells[1], astrocytes[65]	No data	Rat cortical astrocytes[66], mouse astrocytes[65]
EAAT5	No data	Retina[67]	No data	Bipolar cells[67]	No data	No data
xCT, 4F2hc/ x_c^- *	Cortex, cerebellum[68], area postrema, habenular nucleus, subfornical organ, hypothalamus, meninges[69]	Leptomeninges, ventricles, choroid plexus[70]	Neurons of cortex and cerebellum[68], ependymal cells of the lateral wall of the third ventricle[69]	Astrocytes[71], microglia[72], Müller cells[73], immature cortical neurons[74], vascular endothelial cells, ependymal cells[42]	No data	Glioma[75]
VGLUT1	Cortex (layers II–VI), thalamus, some nuclei of the brain stem; vestibular and cochlear nuclei, lateral reticular, external cuneate[76], cerebellum, striatum[43]	Hippocampus, neocortex (layers I–III), cerebellum, entorhinal and piriform cortex, amygdalia, subiculum[43]	Dopaminergic neurons cholinergic neurons GABAergic neurons[43]	Photoreceptors and bipolar cells[43]	No data	Hippocampal, cerebellar and cerebrocortical neurons[77–79]
VGLUT2	Cortex (middle layers), thalamus, most nuclei of brain stem[76], cerebellum, striatum[43]	Cerebral cortex (layer IV), dentate gyrus (granular layer), thalamus, hypothalamus, olfactory bulb, the brain stem[43]	Glutamatergic neurons[76], dopaminergic neurons, GABAergic neurons, cholinergic neurons[43]	Purkinje cells, ganglion cells[43]	No data	Hippocampal, cerebellar and cerebrocortical neurons[78, 79–83]
VGLUT3	Cerebellum, striatum[43]	Hippocampus, neo–cortex, hypothalamus, olfactory bulb, substantia nigra, raphe nuclei, striatum[43]	Dopaminergic neurons cholinergic neurons GABAergic neurons[43]	Glutamatergic neurons, GABAergic neurons, serotonergic neurons, cholinergic neurons, amacrine cells[43]	No data	No data

* xCT denotes mRNA of the light chain of the x_c^- antiporter, 4F2hc-mRNA of the heavy chain of the x_c^- antiporter, x_c^- -xc- antiporter protein.

which involves high Cl^- conductance with relatively low Glu uptake[85].

VGLUTs mediate the accumulation of Glu in secretory vesicles. VGLUT1 knockout mice show progressive neurological deficits, including blindness, incoordination and an enhanced startle response[86–88]. The synaptic vesicles isolated from these mice exhibit a diminished rate of Glu uptake. These animals also showed decreased Glu neurotransmission in hippocampal neurons[87]. The genetic inactivation of VGLUT2 results in perinatal lethality, consistent with the predominant expression of VGLUT2 during embryogenesis and early postnatal development[83, 89]. VGLUT3 is involved in auditory function and mechanical hypersensitivity[90–92].

System x_c^- mediates the uptake of the sulfur-containing amino acid cystine in exchange for Glu at a 1:1 ratio[93]. This system comprises two subunits: 4F2hc, required for cell surface expression, and xCT with typical functional activity. Aside from the primary role of system x_c^-, the maintenance of intracellular GSH levels in astrocytes is essential[94]. After uptake, cystine is reduced to cysteine, which is the primary rate-limiting amino acid in GSH synthesis[95]. Therefore, system x_c^- is involved in the defense against oxidative stress[96]. The Na^+-independent Glu uptake, mediated through the system x_c^-, likely represents only a small fraction of the total astrocyte Glu uptake under physiological conditions.

Mechanism

The Na^+ and K^+ electrochemical gradient is the driving force for Glu transport coupled to EAATs across the cell membrane. This negative membrane potential maintains $[Glu]_i$ at a range 5×10^6-fold greater, compared with the $[Glu]_e$[97]. An accepted model of EAAT action involves the transport of Glu across the cellular membrane coupled to the inwardly directed electrochemical potential gradients of Na^+ and H^+, and the outwardly directed K^+ gradient. The initiation of this process involves the recruitment of Glu and three Na^+ and one H^+ from the extracellular space to an outward-facing conformation of the transporter. The binding of these substrates triggers a conformational change, which adopts an inward-facing conformation of EAATs, followed by cargo release into the cytoplasm of the cell. The subsequent step involves the recruitment of K^+ ions to an EAAT transporter from the cytoplasm, which evokes the return to an outward-facing conformation and release outside the cell[98, 99]. (Figure 2). The disruption of the electrochemical potential reverses Glu transport. Cerebral ischemia, for example, leads to the Na^+/K^+-ATPase function impairment, resulting in a decrease in the ratio of the extracellular Na^+ concentration ($[Na^+]_e$) to the intracellular K^+ concentration ($[K^+]_i$) and/or an increase in $[Na^+]_i/[K^+]_e$[97].

EAATs are also involved in substrate-gated anion conductance, mediated through EAAT4 and EAAT5. The Glu uptake capacity of these molecules is relatively poor. The remaining EAATs also demonstrate anion conductance, with decreasing permeability (GLAST>EAAC1>>GLT-1) and selectivity ($NO3^-$ >I^->Br^->Cl^->F^-). The physiological purpose of anion influx likely compensates for the positive charge influx during Glu transport[100].

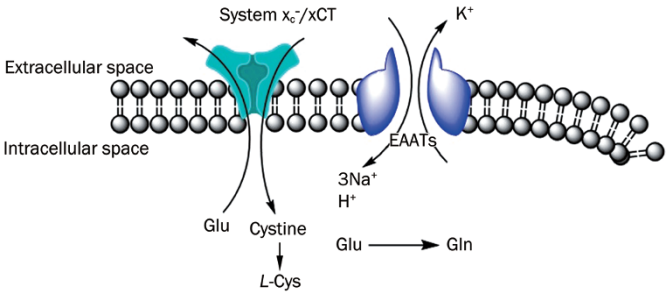

Figure 2. Glu transporters mode of action. The role of the system x_c^-/xCT is to uptake *L*-Cys from synaptic space to intracellular space in change of Glu. The opposite direction of Glu transport mediated by EAATs, depends on co-transport of three Na^+, one proton and one Glu molecule in change of one K^+. EAATs indicates excitatory amino acids transporters; Gln, glutamine; Glu, glutamate; Cys, *L*-cysteine; System xc-/xCT, cystine/glutamate antiporter.

VGLUTs depend on the existence of a vesicular membrane potential gradient, rather than a pH gradient. The proton electrochemical gradient, which exists across the vesicle membrane, is the driving force for Glu uptake. This potential is created by a vacuolar-type ATPase. These transporters are also highly specific for Glu, however the affinity of these molecules is relatively low (K_m=1–3 mmol/L). VGLUTs achieve maximum activity at a low concentration of Cl^- (2–5 mmol/L)[101–105].

The system x_c^- is necessary for cystine uptake. The gradient of high $[Glu]_i$ and low $[Glu]_e$ concentrations is the driving force leading to the cystine import and thus, the intracellular Glu depletion inhibits cystine uptake[93]. The system x_c^- is characterized by Na^+-independent Glu uptake and typically comprises Cl^--dependent glutamate/cystine antiporters. This system shows sensitivity to Cl^- transport blockers, including 4,4′-diiso-thiocyanatostilbene-2,2′-disulfonic acid (DIDS) and furosemide[106]. Human fibroblasts preloaded with [^{3}H]-labeled cystine and deprived of Glu, release cystine, when Glu is added to the incubating medium, indicating the ability of system x_c^- to reverse its manner of action[93] (Figure 2).

Expression

Distinct genes, encoding eukaryotic Glu transporters, and their chromosomal loci are presented in Table 2. The expression of these genes is cell and tissue specific (Table 1).

The expression of EAATs at the mRNA and protein levels does not always occur in parallel, *eg*, GLT-1 mRNA was observed in some hippocampal neurons, but not GLT-1 transporter protein[107]. The enormous loss of GLT-1 protein in the motor cortex in amyotrophic lateral sclerosis (ALS) does not occur with the decrease in the corresponding mRNA[108]. In turn, a nearly simultaneous decrease in both GLT-1 protein and its corresponding mRNA in the rat hippocampus has been observed in transient ischemia[109]. These findings might suggest that the metabolic turnover rates of Glu transporter proteins and their corresponding mRNAs are regulated through

Table 2. Genes encoding Glu transporter proteins and their chromosomal loci in humans and rodents.

Transporter name		Gene	Locus		
Humans	Rodents	name	Humans	Rats	Mice
EAAT1	GLAST	*SLC1A3*	5p13	2q16	15A2;15 3.82cM
EAAT2	GLT-1	*SLC1A2*	11p13-p12	3q31	2 54.13cM
EAAT3	EAAC1	*SLC1A1*	9p24	1q54	19
EAAT4	EAAT4	*SLC1A6*	19p13	7q11	10 39.72cM
EAAT5	EAAT5	*SLC1A7*	1p32	5q35	4
xCT	xCT	*SLC7A11*	4q28-q32	2q26	3 21.72cM
4F2hc	4F2hc	*SLC3A2*	11q13	1q43	19 5.44cM
VGLUT1	VGLUT1	*SLC17A7*	19q13	1q22	7
VGLUT2	VGLUT2	*SLC17A6*	11p14	1q22	7
VGLUT3	VGLUT3	*SLC17A8*	12q23	7q13	10

different mechanisms, depending on cell phenotype, signaling pathways and environmental cues[110].

Regulation

The regulation of the EAATs can be achieved at the level of gene expression and the modulation of the kinetics of their action. The expression and maintenance of functionally active Glu transporters requires both neuronal and non-neuronal factors, *eg*, the induced expression of GLT-1 in cultured astrocytes only occurs in co-culture with neurons. Several factors have been demonstrated to induce GLT-1 expression in astroglial cultures, including cAMP[66], pituitary adenylate cyclase-activating peptide (PACAP, a neuron-derived peptide)[111] and brain-derived neurotrophic factor (BDNF)[112] or the activation of epidermal growth factor (EGF) receptor[113]. Many studies with pharmacological and genetic manipulations imply that the increase of GLT-1 expression might depend on phosphatidylinositol-3-kinase (PI3-K) and nuclear factor kappa B (NF-κB) signaling pathways[114, 115]. NF-κB directly binds to the GLT-1 promoter and thereby regulates its transcription[115]. NF-κB might act as both a transcriptional activator[116] and a repressor[117]. It has been suggested that the GLT-1 promoter region contains distinct NF-κB sites in the proximal and 5′UTR regions, and this transcription factor might differentially contribute to gene activation or repression, depending on the binding site[115]. For example, tumor necrosis factor-α (TNF-α) can inhibit GLT-1 transcription, and this inhibition requires NF-κB[115]. On the other hand, NF-κB, by itself mediates cAMP-, TGF-α, or EGF-induced GLT-1 promoter activation[59, 115].

Methylation of the promoter region is another mechanism to regulate EAAT expression, *eg*, the methylation of the GLT-1 CpG promoter region (a regulatory sequence rich in cytosine and guanine) altering the binding properties of nuclear factors to particular DNA sites. The inhibition of the DNA methyltransferases results in a potent GLT-1 mRNA transcription enhancement, and some endocrine substances can modulate the expression of Glu transporters, *eg*, growth hormone promotes GLT-1 expression in the placenta of mice, while insulin-like growth factor-II (IGF-II) action results in the downregulation of placental EAAT4. At physiological concentrations IGF-II maintains the proper levels of GLT-1, EAAC1, and GLAST transporters. Glu also plays an important role in the regulation of EAATs expression, *eg*, the disruption of the cortical glutamatergic pathways results in the selective and transient down-regulation of GLT-1 and GLAST transporters in the rat striatum and hippocampus, with no changes in EAAC1 expression[118, 119]. The human and murine xCT genes possess 5′ flanking regions containing multiple putative AP-1 binding sites[120, 121]. A presumed NF-κB binding site was also identified in this region, however, there is no direct evidence that this factor activates the transcription of the *xCT* gene[122]. The proximal 5′ flanking region of the murine *xCT* gene also contains four antioxidant response elements (AREs). One of the AREs, located closest to the 5′ end, is involved in induction of xCT promoter activity, associated with the transcription factor Nrf2 and oxidative stress[70]. The hippocampal cell line HT22, treated with the Nrf2 inducer tert-butylhydroquinone (tBHQ), showed a strong increase in *xCT* protein and system x_c^- activity, following the upregulation of the Nrf2 protein level[123]. Moreover, a major increase in xCT protein levels was observed in retroviral-transfected rat astrocyte cultures over-expressing Nrf2[124].

Similarly, tandem amino acid response elements (AAREs) have been observed in the murine *xCT* gene. These regulatory elements are completely conserved among *xCT* 5′ flanking regions in humans, mice, rats and bovines. The AARE located closest to the *xCT* 5′ end binds the transcription factor ATF4. The upregulation of ATF4 intensifies xCT mRNA synthesis with an increase in xCT protein levels and system x_c^- activity in the HT22 hippocampal neuronal cell line, mouse embryonic fibroblasts and the rat PC12 cell line[123]. Both AAREs together mediate *xCT* promoter activation through amino acid starvation[125].

The kinetic regulation of EAATs includes membrane translocation, amino acid phosphorylation, sulfhydryl-based redox reactions, interactions with arachidonic acid (AA), Zn^{2+} or Glu transporter associated proteins (GTRAP) or transporter multimerization[126].

Many factors affect the membrane translocation of Glu transporters. EAAC1-mediated transport is activated through platelet-derived growth factor (PDGF), associated with the redistribution of EAAC1 from the intracellular compartment to the cell surface. Glu also initiates the movement of internalized GLAST to the cell membrane surface and the rapid increase in the maximal transport rate (V_{max}) for Glu uptake[127]. The action of protein kinase C (PKC) might also affect the membrane trafficking of Glu transporters. PKC activation causes the rapid intracellular capture of GLAST, resulting in reduced activity[128]. In addition, EAAC1, expressed in *Xenopus* oocytes, was downregulated in response to PKC activation, associated with the movement of EAAC1 protein from the cell plasma into the intracellular compartment, without any changes in the EAAC1 affinity for Glu[129]. However, in the C6

glioma cell line, PKC activation resulted in EAAC1 mobilization in the plasma membrane, followed by an increase in Glu transport activity[130].

Glu transporter activity might also be affected through Glu-associated proteins. Thus, Glu transporter-associated protein 41 (GTRAP41) and Glu transporter-associated protein 48 (GTRAP48) modulate EAAT4 activity, and the expression of these proteins is associated with an increase in the V_{max} of Glu[131]. EAAC1 cooperates with GTRAP3-18. However, the latter protein action results in a decrease of EAAC1 affinity for Glu and the reduced transport of neurotransmitter mediated through EAAC1[132].

Both GLT-1 and GLAST transporter proteins contain cysteine-associated sulfhydryl groups sensitive to free radical species. The actions of these compounds result in the formation of cystine bridges, thereby inhibiting Glu flux through transporters[133], as demonstrated for superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), NO and peroxynitrite anion ($ONOO^-$)[134, 135]. However, the overexpression of superoxide dismutase 1 protected Glu transporters from inhibition[136].

AA significantly increased substrate-activated currents in oocytes expressing EAAT4[137]. AAs also modulate the actions of other EAATs, *eg*, increasing GLT-1 substrate affinity and reducing the maximal rate of GLAST uptake[138].

Zn^{2+} ions regulate astrocytic EAATs through interactions with transporter proteins. This direct interaction with the GLAST transporter leads to potent and selective inhibition[139]. It has been hypothesized that the Zn^{2+} released during cerebral ischemia might induce excitotoxic neuronal death *via* GLAST inhibition[126].

The activity of Glu transporters might also be regulated through the activation of diverse receptors. For example, Glu uptake in astrocytes is modulated through adrenergic receptors. The norepinephrine (an agonist of α- and β- receptor subtypes) and phenylephrine (an α-receptor agonist) increase the Glu uptake[140]. However, isoproterenol (a β-receptor agonist) decreases Glu uptake in astrocyte primary cultures[141]. Glucocorticoids reduce GLAST and GLT-1 affinities to Glu in astrocytes and thus inhibit Glu transport[142]. Another hormone, melatonin, was shown to stimulate the high-affinity Glu uptake in the retina[143]. The kinetic activity of Glu transporters is also regulated through Glu receptor activation, *eg*, the Glu uptake rate in crude synaptosomes from the mouse cerebral cortex was slightly decreased after treatment with phencyclidine, a noncompetitive antagonist of NMDA receptors. Stronger inhibition was demonstrated through an antagonist of mGluR, (*R*,*S*)-2-amino-3-phosphonopropionic acid[144]. In addition, kainate stimulates high-affinity Glu uptake in the crude synaptosomal preparation[145].

It has been well documented that Cl^- plays a regulatory role in VGLUT activity. The permeant Cl^- increased vesicular Glu uptake at low concentrations, whereas higher concentrations of Cl^- have an inhibitory effect on this uptake[105]. The exact mechanism of the Cl^- effect is unclear. At low concentrations of Cl^-, this regulatory effect has been associated with the presence of a positive allosteric regulatory binding site. The hypothesis explaining the role of high Cl^- concentrations is associated with a buffering role. VGLUT bioenergetics primarily rely on Δψ, the main driving force for Glu accumulation. The high luminal concentration of Cl^- will buffer H^+, stimulating V-ATPase and thus decrease Δψ and increase ΔpH, which might consequently reduce VGLUT activity[146]. The stimulation of VGLUT through Cl^- is completely inhibited through keto acids and pyruvate and acetoacetate[147]. Moreover, fatty acids, amino acids, quinolines, azodyes, alkaloids, kynurenic acid and quinoline-related compounds have been reported to inhibit VGLUTs. AAs and polyunsaturated fatty acids, released under pathological conditions, such as ischemia, also inhibit the vesicular uptake of Glu[92]. The treatment of cultured neurons with subtoxic levels of NMDA resulted in VGLUT1 mRNA upregulation[148].

Both, extra- and intracellular levels of Glu are critical for the cystine uptake through the system x_c-. Thus, the pathways that govern Glu concentrations might affect the activity of system x_c-. EAATs participate in the regulation of system x_c-[122]. An α-aminoadipate (a molecule, which can be transported through system x_c- and a metabolite of lysine metabolism in the brain) inhibits this system[149]. In addition, acidosis might also affect system x_c-. A study on cultured human fibroblasts revealed that extracellular acidosis (pH=6.5) inhibits the transport of cystine *via* system x_c-, while the Glu transport through this exchanger is much less affected[150]. Acidosis in the brain, subsequent to cerebral ischemia, is often associated with prominent lactate accumulation[151]. A study using rat cortical astrocytes indicated the inhibition of system x_c-activity through lactate[152]. However, a study using the hippocampal cell line HT22 did not show any influence of the latter compound on x_c-activity[153].

Glutamate transporters in brain ischemia

The predominant mode of Glu release depends on the strength of the injury. In fact, the reversal of Glu transporters has been shown to play a major role in the release of Glu after severe ischemia[154]. However, other pathways are also involved in Glu efflux, *eg*, gap-junction, hemichannel-mediated and P2X channel-dependent release from astrocytes[155] and release from neurons through exocytosis[156, 157].

The neurotoxicity of Glu in the brain ischemia has been associated with the reversal of Glu transporters, presumably neuronal Glu transport[154]. The depletion of the energy supply, leads to the disruption of transmembrane electrochemical gradients, resulting in decreased Glu uptake or Glu efflux *via* uptake reversal[31]. Glu transport reversal, due to ATP depletion, has been demonstrated in primary astrocyte cultures[158] and slice preparations[159].

In vitro models of ischemia

Several data indicate the up-regulation of EAATs following hypoxia *in vitro*, *eg*, rat pheochromocytoma (PC12) cell exposure to hypoxia resulted in the significant up-regulation of EAAC1 and GLT-1 expression, without any influence on GLAST expression at both the mRNA and protein levels.

Moreover, an increase in Na^+-dependent Glu uptake was shown in response to hypoxia[160]. In addition, exposure to hypoxic conditions resulted in a significant increase in the EAAC1 mRNA level in the C6 rat glioma cell line and the murine GLT-1 mRNA level in GT1-7 cells in an RNAse protection assay. In C6 hypoxic cells a decreased uptake of *D*-aspartate from incubating medium was shown despite the increased expression of Glu transporters[161]. One may speculate, that this reduction in the *D*-aspartate uptake may results from the reversed mode of Glu transporters action. These results reflect an attempt to maintain ion and Glu homeostasis under restricted energy and oxygen supply[162–164].

The results of studies using an oxygen-glucose deprivation (OGD) model of *in vitro* global ischemia suggest that Glu transporters reduce the $[Glu]_e$ during the early stages of ischemia. However, in the later phases, Glu transporters become the source of extracellular Glu, acting in a reversed manner. Numerous studies using different EAAT blockers were performed to explain this phenomenon, *eg*, *L*-trans-pyrrolidine-2,4-dicarboxylic acid (PDC), a competitive, transportable inhibitor of GLAST, GLT-1, EAAC1 and EAAT4, and a non-transportable inhibitor of EAAT5, and *DL*-threo-β-benzyloxyaspartate (*DL*-TBOA), a potent competitive inhibitor of Na^+-dependent Glu/aspartate transporters (including GLAST, GLT-1, and EAAC1), significantly augmented Glu efflux from cerebrocortical slice cultures during OGD for 30 min. These results suggest that the activity of EAATs was in forward operation. However, after a 60 min incubation with PDC under OGD conditions, Glu accumulation was reduced, suggesting a transition into the reverse mode of operation of Glu transporters[165]. This evidence corresponds with the results of another experiment, where the incubation of adult corticostriatal slices with *DL*-TBOA during OGD for 30 min did not attenuate increases in Glu[166]. In turn, hippocampal slices incubated with *DL*-TBOA under OGD conditions for the same period of time showed the reduced OGD-induced damage of CA1 pyramidal neurons[167]. Similarly, the *DL*-TBOA-treatment of cultured astrocytes and neurons subjected to ATP depletion significantly reduced Glu release[168]. The 30-min application of OGD to adult corticostriatal slices resulted in a significant increase in the Glu level, whereas incubation with dihydrokainate (DHK), a selective inhibitor of GLT-1, attenuated this increase[166]. Similar results were obtained in studies involving acute hippocampal slices[169]. Moreover, the virus-induced increased expression of GLT-1 significantly augmented the vulnerability to Glu in hippocampal slice cultures[170]. However, there are also data suggesting that under ischemic conditions inhibitors of Glu transporters may differ in some respects of their action. For example, PDC in high concentrations can induce the release of Glu from astrocytes and neurons, whereas *DL*-TBOA not[171]. Thus, PDC may affect ischemia-induced Glu efflux mediated by Glu transporters.

In addition, an increase in GLAST and GLT-1 expression was demonstrated after incubation of the hippocampal slice culture with glial cell line-derived neurotrophic factors (GDNF). The exposure of the GDNF-treated hippocampal slice culture to OGD for 30 min resulted in an increase in OGD-induced cell death[172]. These results indicate the involvement of GLT-1 in OGD-induced extracellular Glu accumulation and the reversed transport of this neurotransmitter via Glu transporters is involved in OGD-induced Glu accumulation.

In vivo models of ischemia

GLT-1 expression in the ischemic brain

Many studies have demonstrated the downregulation of GLT-1 expression after cerebral ischemia in rodents. A significant reduction in GLT-1 mRNA expression has been demonstrated in the CA1 hippocampal area at 6, 12, and 24 h following transient global brain ischemia in rats[173]. A similar decrease in GLT-1 mRNA expression was observed in the ipsilateral hippocampus at 24 h after middle cerebral artery occlusion (MCAO) in mice[174]. This result was consistent with the GLT-1 *in situ* hybridization assay, showing a significant decrease in GLT-1 mRNA expression in the CA1 hippocampal region from 1 d after bilateral common carotid arteries occlusion (BCCAO) in rats up to 21 d of reperfusion. In the CA3 hippocampal region, a decrease in the GLT-1 mRNA expression level was recorded on d 1[175]. Consistent results were obtained in the Western blot analysis, demonstrating a significant decrease in the GLT-1 protein level in the rodent hippocampus at the early (6 h) and later (3 d) phases of reperfusion after transient global brain ischemia[173, 176, 177]. Similarly, an immunohistochemical study revealed a significant decrease in GLT-1 in the CA1 hippocampal region from day 2 to 4 after ischemia-reperfusion in the BCCAO model in rodents[175]. A histopathological assessment revealed the progressive loss of neurons in the CA1 hippocampal region from 3 d of reperfusion, followed by 5 and 7 d after ischemia. Altogether, these results suggest that GLT-1 dysfunction might contribute to neuronal death in the gerbil hippocampus following BCCAO[173, 176, 177]. However, a significant increase in immunoreactivity has been demonstrated during the early stage (0.5–12 h) after reperfusion in the CA1 hippocampal subfield in global ischemia in gerbils[178]. In addition, a progressive rise in GLT-1 immunoreactivity was observed from d 1 to 21 after global ischemia in the CA3 hippocampal region in rats[175]. Inconsistencies in these results might reflect the use of various analytical methods and animal models of ischemia.

In the ipsilateral cortex a significant decrease in GLT-1 mRNA expression was observed at 24 and 72 h after MCAO in rodents[174, 179]. An immunohistochemical analysis confirmed these latter findings, showing a parallel significant reduction in GLT-1 protein levels in the rodent cerebral cortex[174, 178]. In the rat striatum, the GLT-1 expression level was reduced at 1 h after transient global ischemia and remained decreased for 6 h after ischemia, returning to basal levels after 24 h of reperfusion[176]. Surprisingly, in the rat subcortical white matter an increase in GLT-1 expression was demonstrated at 1 and 3 d after MCAO using immunohistochemistry and Western blot analysis[180]. These results show that post-ischemic changes in the expression of GLT1 depend on the brain structure location.

EAAC1 expression in the ischemic brain

The results of studies concerning EAAC1 expression are inconsistent. In the murine hippocampus, EAAC1 mRNA and protein levels did not change at 24 h after MCAO[174]. Similarly, no changes in EAAC1 expression in the rat CA1 hippocampal region were observed after1 to 24 h at 3 and 7 d following transient global ischemia in rats[176]. However, a decrease in EAAC1 protein expression in the CA1 area at 30 min after BCCAO was demonstrated in gerbils, followed by transient enhancement at 3–12 h after reperfusion and a final reduction at 24 h after ischemia, remaining reduced for 10 d[177, 178].

In addition, the data regarding cortical EAAC1 expression is ambiguous. Rao *et al*[181] reported a significant decrease in the mRNA and protein levels at 24 and 72 h after rat MCAO, while Ketheeswaranathan *et al*[174] did not observe any changes in the EAAC1 expression at 24 h in the same ischemia model in mice.

In the rat striatum, the EAAC1 protein level was basically undetectable at 1 h to 7 d after transient global ischemia[176]. In the rat subcortical white matter, a slight increase in the EAAC1 protein expression was shown at 1 and 3 d after MCAO[180]. These findings might suggest that EAAC1 expression in brains subjected to ischemia depends on the experimental paradigm (global *vs* focal ischemia) and is structure specific (striatum *vs* white matter).

GLAST expression in the ischemic brain

No changes in the GLAST mRNA levels were observed in the hippocampus, cerebral cortex and striatum in rodents at 6 to 24 h following transient global or focal ischemia[173, 174]. An immunohistochemical study showed a markedly increased GLAST level in the CA1 hippocampal area at 0.5 to 12 h after BCCAO, followed by a decrease in expression at 24 h, which remained decreased at 10 d after reperfusion[178]. No significant changes in the GLAST protein level have been determined in the CA1 hippocampal area between 6 h and 7 d after a transient global ischemia in rats nor gerbils[173, 176, 177]. In addition, global and focal brain ischemia did not significantly influence cortical GLAST mRNA and protein expression between 6 h and 7 d in rodents[174, 181]. In the rat subcortical white matter, MCAO caused a significant increase in GLAST protein expression on days 1 to 3 according to the immunohistochemical and Western blot analyses[180]. These results suggest that changes in GLAST expression depend on brain structure and time after reperfusion.

VGLUTs in ischemic brain.

Little is known about changes in VGLUT proteins and mRNA after brain ischemia in rodents. VGLUT1 was increased in the caudate-putamen and cortex within 3 d after reperfusion and then decreased at 7 d after MCAO in rats. VGLUT2 and VGLUT3 were drastically reduced within this time period[182]. In addition, a significant decrease in the VGLUT-2 protein level was recorded under excitotoxic conditions *in vivo* and *in vitro* following MCAO[183]. This change was associated with increased calpains activity, as these enzymes play a key role in neuronal death following excitotoxic or ischemic insults[184]. Calpains are also activated following MCAO in striatum and cerebral cortex, leading to neuronal death[185]. Increased VGLUT 2 and 3 activity was observed in reactive astrocytes of the ischemic corpus callosum and cortex. In the same study, it was shown that GLT-1 expression was parallel to VGLUT1 expression in the caudate-putamen, suggesting the potential interplay between these two transporters in the regulation of Glu levels[182]. However, other studies did not show any changes in VGLUT1 expression after focal cerebral ischemia, while VGLUT2 and VGLUT3 mRNAs were downregulated[186]. Another study on focal cerebral ischemia demonstrated reduction in mRNA level of VGLUT1-3 in the hippocampus and cerebral cortex of young rats[187], suggesting an age-dependent modification in VGLUTs expression. Indeed, in young animals the level of VGLUT1 protein was reduced in the hippocampus and cortex, whereas VGLUT2 protein level was increased in these structures, but reduced in the dentate gyrus. Interestingly, the VGLUT2 protein level in adult animals was lower after ischemia in CA3 hippocampal region, dentate gyrus and cortex, but higher in the CA1 compared with the control. VGLUT3 mRNA was reduced in all areas after focal ischemia[187]. These findings suggested that changes in the expression of VGLUT proteins, following focal cerebral ischemia, are age-related.

In conclusion, brain ischemia modulates the expression of the Glu transporters in CNS cells (Table 3). However, there are some inconsistencies among results of different studies, potentially reflecting the diverse animal species, models of ischemia and types of analytical methods applied in these experiments. Thus, it is difficult to draw reliable conclusions, and further investigations are required.

Role of Glu transporters in ischemia

Several studies using cerebral ischemia animal models indicate a protective role for Glu transporters in the reduction of $[Glu]_e$ levels, decreased cell loss and infarct volume and improvements in behavioral recovery.

A whole-cell patch-clamp recording of hippocampal CA1 astrocytes in post-ischemic slices demonstrated a significant reduction in the maximal amplitude of Glu transporter currents at 6 to 24 h after global ischemia compared with the control[173]. Rats infected with adeno-associated viral vectors, expressing the rat GLT-1 cDNA (AAV-GLT1) showed a significant decrease in ischemia-triggered Glu overflow in a microdialysis assay. This infection also resulted in a significant decrease of brain infarct and DNA fragmentation in the region of AAV-GLT1 injection and an improvement in post-ischemic behavioral recovery[188]. In contrast, significantly higher Glu concentrations were measured in the dialysates collected from the C57BL/6 mice lacking GLT-1 gene expression after BCCAO for 5 min. GLT-1 knockouts also displayed more pronounced delayed neuronal death in the CA1 hippocampal region. Surprisingly, the Glu levels measured in wild-type mice were significantly higher compared with GLT-

Table 3. Expression of the Glu transporters in the ischemic brain in rodents.

Glu transporter	Time after reperfusion	Hippocampus		Cerebral cortex		Striatum		Subcortical white matter	
		mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein
GLT-1	≤24 h after reperfusion	↓ (CA1) global ischemia[193] ↓ Focal ischemia[174] ↓ (CA3) global ischemia[175]	↓ (CA1) global ischemia[173, 176] ↓ Global ischemia[177] ↓ (CA1) global ischemia[180] ↑ (CA1) global ischemia[180] ↑ (CA3) global ischemia[175]	↓ Focal ischemia[174, 178]	↓ Focal ischemia[174, 178]	↔ Focal ischemia[174]	↓ Global ischemia[176]	No data	↑ Focal ischemia[179]
	>24 h after reperfusion	↓ (CA1) global ischemia[175] ↔ (CA1) global ischemia[173] ↔ (CA1) global ischemia[176]	↓ (CA1) global ischemia[175] ↑ (CA3) global ischemia[175] ↔ (CA1) global ischemia[173] ↔ (CA1) global ischemia[176]	↓ Focal ischemia[174, 178]	↓ Focal ischemia[174, 178]	No data	No data	No data	↑ Focal ischemia[179]
EAAC1	≤24 h after reperfusion	↔ Focal ischemia[174]	↔ Focal ischemia[174] ↔ (CA1) global ischemia[176] ↓ (CA1) global ischemia[176, 180]	↓ Focal ischemia[181] ↔ Focal ischemia[174]	↓ Focal ischemia[181]	No data	Undetectable[176]	No data	↔ Focal ischemia[179]
	>24 h after reperfusion		↔ (CA1) global ischemia[176] ↓ (CA1) global ischemia[176, 180]	↓ Focal ischemia[181]	↓ Focal ischemia[181]	No data	Undetectable[176]	No data	↔ Focal ischemia[179]
GLAST	≤24 h after reperfusion	↔ Global ischemia[173] ↔ Focal ischemia[174]	↑ (CA1) global ischemia[180] ↓ (CA1) global ischemia[180] ↔ (CA1) global ischemia[173, 176] ↔ global ischemia[177]	↔ Global ischemia[173] ↔ Focal ischemia[174, 181]	↔ Focal ischemia[174, 181]	↔ Global ischemia[173] ↔ Focal ischemia[174]	↔ Global ischemia[176]	No data	↑ Focal ischemia[179]
	>24 h after reperfusion	No data	↓ (CA1) global ischemia[180] ↔ (CA1) global ischemia[173, 176] ↔ global ischemia[177]	↔ Focal ischemia[181]	↔ Focal ischemia[174, 181]	No data	↔ Global ischemia[176]	No data	↑ Focal ischemia[179]

↑ – significantly higher compared to control; ↓ – significantly lower compared to control; ↔ – no significant change compared to control.

1-lacking mice after 20 min of ischemia. In addition, acute neuronal death in the CA1 hippocampal region was present in wild-type mice[189]. Rao and co-workers[178] conducted experiments using GLT-1 antisense oligodeoxynucleotides infusions in cerebrospinal fluid (CSF), followed by 1 h transient MCAO in rats. The results showed an increased infarct volume, neurological deficit and mortality compared with GLT-1 sense/random oligodeoxynucleotide-infused control rats. The administration of EAAC1 antisense oligodeoxynucleotides did not influence the above parameters[181]. Similarly, the GLT-1 heterozygous knockout mice (GLT-$1^{+/-}$), subjected to MCAO, displayed significantly increased brain swelling, and the intraperitoneal administration of DHK to these animals intensified this effect[190].

The C57BL/6 mice, lacking EAAT4 expression, did not display any reduction of the Purkinje cell number in the cerebellum after 5-min ischemia. In contrast, in C57BL/6 mice, lacking GLAST expression, a significant loss of the Purkinje cells was demonstrated. This loss was associated with EAAT4-low zones of cerebellum in GLAST mutants, suggesting that in cooperation with EAAT4, GLAST participates in preventing the excitotoxic damage of the cerebellum following ischemia[191].

Human data

Data from studies on human tissue are sparse because of several limitations. Analyzed patients differed in terms of age, location of the infarct and time of death after stroke.

An immunohistochemical study of brains obtained from adult patients with ischemic stroke, showed a significant reduction of both GLAST and GLT-1 cortical expression in the lesion within 24 h after stroke onset. However, a large increase in the quantity of the white matter GLAST-positive cells was observed within 24 h in the lesion and this increase was maintained for months in both adjacent and remote infarction areas. During the first week after stroke (*eg*, ramified and amoeboid), microglia expressed GLAST and subsequently astrocytes were predominantly GLAST-positive, potentially reflecting the neuroprotective potential of microglia and astrocytes after brain ischemia[192].

Data on the glutamatergic system from studies performed in stroke patients are limited. In persons with large brain infarcts, the Glu concentration in plasma and CSF was significantly increased. Moreover, the Glu levels in plasma and CSF were higher in patients with greater neurological deficits[193, 194].

Recently, it has been suggested that some individuals might be more susceptible to stroke severity. Mallolas and co-workers[195] have shown the GLT-1 promoter polymorphism (an A-to-C change at -181 bp) is associated with higher Glu plasma levels after stroke and with a higher risk of early neurological deterioration. This polymorphism is associated with the downregulation of GLT-1 expression after stroke[195].

Modulation of Glu transporters and brain tolerance to ischemia

Preconditioning induces brain tolerance to severe episodes of ischemia. This neuroprotective process can be mediated either through smaller amounts of Glu released into the synaptic cleft or increased Glu uptake by EAATs. Ischemic preconditioning, *ie*, short episodes of ischemia, downregulated NMDA and AMPA receptors, thereby ameliorating excitotoxicity[196–198]. The introduction of 5-min preconditioning with OGD prior to 1 h exposure to OGD resulted in a significant reduction of neuron damage. This reduction might reflect the increased expression of GLT-1 protein[199]. However, depending on the type of animal model of ischemia and preconditioning stimulus used, reports on Glu transporter expression and activity are inconsistent, *eg*, 10 min of ischemic preconditioning caused the upregulation of GLT-1 and EAAC1, but not GLAST, expression in rats[200]. Cortical spreading depression (CSD) in the same species resulted in the downregulation of GLAST and GLT-1 at 1–7 d after preconditioning with CSD[201]. In contrast, hypoxic preconditioning after a 3 h exposure to 8% oxygen in newborn rats, followed by severe brain ischemia evoked at 24 h later, diminished brain injury in some regions, namely in the cortex, striatum and hippocampus. In addition, the expression of GLAST was unaffected, while the expression of GLT-1 either increased in the cortex or decreased in the striatum[202].

Recently, it has been reported that cerebral ischemic preconditioning caused significant upregulation of GLT-1a in the CA1 hippocampus area in rats, whereas severe brain ischemia, without preconditioning, caused the down-regulation of transporter expression and a corresponding increase in the concentration of Glu and cellular damage. This downregulation of GLT-1a was prevented when preconditioning preceded long-term brain ischemia, and this beneficial effect was inhibited through the intracerebroventricular administration of GLT-1a antisense oligodeoxynucleotides. These findings might suggest that GLT-1a participates in the induction of brain tolerance[203]. Data from *in vitro* experiments suggest that the role of EAATs in ischemic tolerance of the brain is indirect. In cortical cultures exposed to low-oxygen concentrations, ischemic tolerance was associated with the up-regulation of GLT-1 and EAAC1[204]. The co-culture of cortical neurons with astrocytes demonstrated the down-regulation of GLT-1 after OGD preconditioning, associated with the increased resistance of these co-cultures to more severe ischemic episodes through OGD[205]. Mild ischemia might reverse neuronal Glu transport through EAAC1, while a severe ischemia episode, causing brain damage, might also affect glial transporters, including GLT-1. Thus, inducing the overexpression of GLT-1 could also be malicious under some conditions[206].

Pharmacological preconditioning with β-lactam antibiotics changes the expression and activity of EAATs[204, 207, 208]. Rothstein and co-workers[208] discovered that β-lactam antibiotics, including ceftriaxone (CEF), increase the uptake of excessive Glu through the stimulation of the expression of GLT-1 but not of other Glu transporters (GLAST, EAAC1 or EAAT4). In an *in vitro* model of dissociated embryonic cortical cultures, pretreatment for 48 h with CEF before 1 h OGD, the number of death cells decreased of 60%. CEF also reversed the effect of the Glu transporter inhibitors, threo-β-hydroxyasparate (THA) and TBOA, which overload the synaptic cleft with Glu. Treatment with CEF in a dose-dependent manner protected neurons from the harmful effects of THA or TBOA, which reduce $[Glu]_e$. Another study reported that pretreatment for 48 h with CEF before focal cerebral ischemia or OGD, induced neuroprotective effects. In MCAO, pretreatment with CEF upregulated the mRNA and protein levels of GLT-1 and EAAC1 and reduced the infarct volume in rats[204]. The neuroprotective mechanism underlying the effects of CEF depends on the NF-κB pathway and GLT-1 promoter activation[209].

Another plausible attempt to modulate Glu transport within the CNS involves the x_c^- system. Upregulation of x_c^- and activation of the antioxidant defense might diminish isch-

emic damage, even more than EAATs modulation. Oxidative stress during ischemia induces the phosphorylation of Nrf2, resulting in the increased transcription and translation of x_c- system elements after translocation into the nucleus and binding to ARE[70]. It was recently reported that CEF produced an increased Nrf-2 and xCT expression in HT22 hippocampal cells. *In vitro* studies have shown the induction of x_c-, with the simultaneous inhibition of EAATs, exacerbated Glu-induced cell death[210–212], suggesting potential cooperation between EAATs and the x_c- system. The data from *in vivo* studies support the idea of neuroprotective activity of the x_c- system. Mice exposed to hypoxic preconditioning showed the increased expression of x_c-, particularly in the hippocampus[213]. In this context, it is reasonable to speculate that in severe brain ischemia, preceded by pretreatment with CEF, the up-regulation of both EAATs and x_c- might prevent excitotoxicity and inhibit the deactivation of membrane xCT[214].

Recent data suggest that the administration of amitriptyline, a tricyclic antidepressant drug, before transient MCAO remarkably reduced infarct volume and neurological deficits and significantly reduced the levels of Glu, Asp, and Gly, but augmented the level of GABA and Tau in rats[215, 216]. Amitriptyline might enhance the expression and activity of Glu transporters. This drug also increases IκBα phosphorylation and NF-κB p65 translocation to the nucleus. The injection of Ro106-9920 (NFκB inhibitor) prevented this translocation, inhibited GLAT and GLT-1 upregulation and restored EAAC1 expression in rats[217]. However, contrary to previous reports, an *in vitro* study using amitriptyline showed the inhibition of EAAC1 activity and enhanced Glu neurotransmission[218].

Riluzole, the drug approved for ALS treatment, is another compound with confirmed modulatory activity on Glu transporters. Riluzole, both significantly increased Glu uptake *in vitro* and *in vivo*. The mechanism underlying the action of riluzole are likely associated with changes in the affinity of GLAST, GLT-1, and EAAC1 to Glu[219]. However, it was also reported that riluzole elevates the expression and activity of GLT-1, but no other transporters, in striatal astrocytes. In the MCAO model, a single dose of riluzole, injected up to 3 h after reperfusion, reduced the infarct volume 75% and improved neurological deficits[220]. Moreover, the *in vitro* model of ischemia, OGD, showed that small doses of riluzole prevented neuronal death[221].

N-acetylcysteine (NAC) is widely used as a mucolytic agent and in paracetamol intoxification. This compound contains sulfhydryl groups and acts as a free radical scavenger. NAC is also a membrane permeable cysteine precursor[222–224] that might enhance the activity of system x_c- and thus increase GSH synthesis. These results improve the oxidative status in nervous tissue[225]. It has been demonstrated that the administration of NAC after cerebral ischemia exhibits neuroprotective effects in rats[226–228]. However, the intensification of x_c- might increase $[Glu]^-_e$, enhancing excitotoxicity[225]. NAC also reduces oxidative stress in the neurons of EAAC1-deficient mice subjected to MCAO. EAAC1 possesses the most potent ability to transport cysteine into neurons, but because NAC is membrane permeable, this compound can enhance GSH synthesis without the mediation of EAAC1[229–231]. There are no reports about the direct modulation of these transporters through NAC; however, it is reasonable to speculate that the enhanced activity of system x_c- and thus increased $[Glu]_e$ could drive the activity of EAAC1.

Recent studies have shown the interesting activity of maslinic acid (MA), a natural triterpene and ingredient of *Olea europaea*. The administration of MA before MCAO was neuroprotective, reduced the infarct volume, improved neurological scores and enhanced the expression of GLT-1 at the protein and mRNA levels in rats. The observed enhanced expression of GLT-1 (mRNA and protein) was likely mediated through NFκB suppression. Similarly, MA promoted neuron survival at high Glu concentrations what might be correlated with an enhanced expression of the Glu transporters, GLAST and GLT-1, *in vitro*[232, 233].

Conclusions

Despite many intensive studies, the pharmacotherapy and prophylaxis of stroke remains unsatisfactory. The only registered drug for the treatment of ischemic stroke is the recombinant tissue plasminogen activator. Unfortunately, the use of this drug is limited by many contraindications, including a narrow therapeutic time window of 4.5 h. Moreover, there is no specific stroke-dedicated prophylaxis. Neuroprotective drugs are also lacking. Thus, there is a need to identify and introduce into routine clinical practice new strategies that would facilitate a decrease in ischemia-induced brain injury. One of the attractive ideas is the induction of brain tolerance to ischemia. Another interesting research area, in the context of brain ischemia, is the modulation of the glutamatergic system, as Glu plays an important, but harmful, role in ischemia-induced cerebral injury. Little is known about the potential significance of Glu transporters and their modulation in the induction of brain tolerance to ischemia. The results of studies using *in vitro* and *in vivo* models of brain ischemia indicated that this approach is promising. Some of the strategies modulating Glu transporters have shown significant protection of the nervous tissue after ischemia. Interestingly, some of pharmacological modulators of Glu transporters are drugs registered and used in clinical practice, *eg*, β-lactam antibiotics, NAC, amitriptyline, and riluzole. However, stroke is not an indication for the use of these drugs. In summary, the results of many studies have provided convincing data that the modulation of Glu transporters is a promising to induce brain tolerance to ischemia. The opportunity to increase resistance to cerebral ischemia is attractive to clinicians and patients. Particularly, persons showing high-risk stroke could targets for this type of therapy. However, a mechanism for avoiding stroke remains unknown, but perhaps a decrease in stroke-related brain injury can be achieved.

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